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Safety Evaluation in Chickens of Candidate Human Vaccines Against Potential Pandemic Strains of Influenza

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SUMMARY. Two candidate formalin-inactivated vaccines, made from high-growth reassortant viruses with the HA and NA genes from avian viruses in a background of genes derived from A/Puerto Rico/8/34 (PR8), were prepared against H5N1 and H9N2 subtypes (designated as H5N1/PR8 and H9N2/PR8, respectively). These viruses bear the genotypes, antigenicity, and attenuation in mouse models that are desirable in candidate vaccines. The pathogenicity of the newly generated avian-human reassortant vaccine viruses was also evaluated in chickens. Neither H5N1/PR8 nor H9N2/PR8 were highly pathogenic for chickens. No clinical signs, gross legions, or histological lesions were observed in chickens that were administered H5N1/PR8 either intranasally (i.n.) or intravenously (i.v.), and virus was not detected in oropharyngeal or cloacal swabs. When H9N2/PR8 was administered i.n., no clinical signs, gross lesions, or histological lesions were observed and no virus was detected in cloacal swabs. However, virus was isolated at low titer from oropharyngeal swabs of all eight chickens. Although no clinical signs were observed when H9N2/PR8 was administered i.v., mild tracheitis was seen in one of two chickens. Moderate amounts of antigen were observed in tracheal respiratory epithelium, and low titers of virus were recovered from oropharyngeal and cloacal swabs of some chickens. In summary, both reassortant vaccine viruses replicated poorly in chickens. These studies suggest that these candidate vaccine viruses carry a low risk of transmission to chickens.

RESUMEN. Evaluación de la seguridad en pollos para vacunas candidatas para uso en humanos contra cepas de influenza potencialmente pandémicas.

Dos vacunas inactivadas con formalina preparadas contra los subtipos H5N1 y H9N2 (designadas como H5N1-PR8 y H9N2-PR8, respectivamente), fueron elaboradas a partir de virus reordenados de multiplicación elevada, con genes HA y NA provenientes de virus aviares en un conjunto de genes derivados del virus A/Puerto Rico/8/34 (PR8). Estos virus poseen los genotipos de antigenicidad y atenuación en modelos de ratones que los hacen deseables como candidatos para vacunas. La patogenicidad de los nuevos virus vacunales reordenados también fue evaluada en pollos. Ninguno de los dos virus, H5N1/PR8 o H9N2/PR8, fue altamente patógeno para los pollos. No se observaron signos clínicos, lesiones macroscópicas o lesiones histológicas en los pollos a los que se les administró H5N1/PR8 por vía intranasal o intravenosa. Tampoco se detectó virus en hisopos orofaríngeos o cloacales. Cuando se administró el virus H9N2/PR8 por vía intranasal no se observaron signos clínicos ni lesiones macroscópicas o histológicas y tampoco se detectó virus en hisopos cloacales, sin embargo, el virus se aisló con un título bajo de hisopos orofaríngeos de los 8 pollos inoculados. Aunque no se observaron signos clínicos cuando el virus H9N2/PR8 fue administrado por vía intravenosa, se observó traqueítis leve en uno de dos pollos. Se observaron cantidades moderadas de antígeno en el epitelio respiratorio de la tráquea y obtuvieron títulos bajos de virus a partir de hisopos orofaríngeos y cloacales de algunos pollos. En resumen, las dos vacunas con virus reordenados se replicaron en bajo nivel en pollos. Estos estudios sugieren que estos virus vacunales candidatos presentan un riesgo bajo de transmisión para los pollos.

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Key words: chickens, influenza vaccine, pandemic vaccine, poultry

Abbreviations: AGP = agar gel precipitation; CID_{50} = 50% chicken infective dose; EID_{50} = 50% egg infectious dose; G1 = A/quail/Hong Kong/G1/97 (H9N2); G9 = A/chicken/Hong Kong/G9/97 (H9N2); HA = hemagglutinin; HK491 = A/Hong Kong/491/97; i.n. = intranasally; i.v. = intravenously; Korea group = A/duck/Hong Kong/Y439/97 (H9N2); NA = neuraminidase; p.i. = postinfection; PR8 = A/Puerto Rico/8/34; SEPRL = Southeast Poultry Research Laboratory; SPF = specific pathogen free; WPR = white Plymouth Rock chickens; wt = wild type

The natural hosts and reservoir for influenza A viruses are aquatic birds that can transmit these viruses to poultry. In 1997, an avian H5N1 influenza virus transmitted from chickens to humans in Hong Kong and resulted in 18 people being hospitalized and six deaths (2,10). An H5N1 influenza virus has reappeared in chickens in Hong Kong in 2001 and in 2002, although no human cases have been reported to date. H9N2 viruses were isolated from avian species in China as well as in North America prior to 1990 and were widespread in Hong Kong and China by 1997, where they cocirculated with H5N1 viruses (3,4). Three lineages of H9N2 influenza viruses have been isolated in avian species in southeastern China, represented by A/quail/Hong Kong/G1/97 (H9N2) (G1:G1 group), A/chicken/Hong Kong/G9/97 (H9N2) (G9:G9 group), and A/duck/Hong Kong/Y439/97 (H9N2) (Korea group) (4,5). G1-like viruses were isolated from two children with mild febrile illnesses in Hong Kong and from five patients with influenza-like illnesses in southeastern China in 1999 (6,7,12).

The observations that avian H5N1 and H9N2 influenza A viruses infected humans in Hong Kong and Southern China and continue to circulate in waterfowl in the region raise concerns that these subtypes have the potential to cause a human pandemic (8,14).

Currently the only licensed human influenza vaccines in the United States are formalin-inactivated vaccines prepared from seed viruses containing hemagglutinin (HA) and neuraminidase (NA) genes from epidemic strains in a background of internal genes derived from the vaccine strain, A/Puerto Rico/8/34 (H1N1) (PR8). Such vaccines are not available for the prevention of human infections by avian influenza viruses. To prepare for potential pandemics due to these avian influenza viruses, we produced two high-growth reassortant vaccine seed viruses that bear H5N1 and H9N2 subtype surface glycoproteins. Formalin-inactivated vaccines prepared from these viruses have been tested in a mouse model for their safety and efficacy (1,9). Although these vaccines demonstrated desirable characteristics such as antigenicity and attenuation in mice, the newly generated avian–human reassortant viruses could have a negative impact on animal agriculture if transmitted back from humans to poultry. Therefore, in this study, the pathogenicity of vaccine candidate viruses was evaluated in chickens.

MATERIALS AND METHODS

Chickens and housing. Four-week-old white Plymouth Rock (WPR) and white leghorn chickens were obtained from specific pathogen free (SPF) stocks maintained at the Southeast Poultry Research Laboratory (SEPRL), Athens, GA. All chickens were housed in negative pressure stainless steel isolation cabinets with continuous light exposure. Water and feed were provided *ad libitum.* All experiments were accomplished in a biosafety level 3 agriculture facility at the SEPRL under the guidance of the Institutional Animal Care and Use Committee.

Viruses. PR8 was obtained from Dr. Roland Levandowski, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD. Reassortant viruses H9N2/PR8 and H5N1/PR8 and transfectant PR8 were generated as previously described (1,9). All viruses including A/Hong Kong/491/97 (HK491) and A/chicken/Hong Kong/G9/97 (G9) were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs.

Experimental design. Ten WPR chickens were inoculated with $10^{6.0}$ 50% egg infectious dose (EID_{50}) in 0.1 ml intranasally (i.n.) or the standard 0.2 ml of 10^{-1} dilution of stock virus intravenously (i.v.). Eight chickens per group were used for pathotyping; oropharangeal and cloacal swabs were collected from each chicken on day 3 postinoculation (p.i.), and all samples were inoculated into embryonated eggs for virus isolation. Chickens were observed for 14 days, and serum samples were harvested and tested for evidence of seroconversion by agar gel precipitation (AGP). Two chickens per group were necropsied on the day of death or were euthanized on day 3 p.i. Tissues were collected and processed for routine histologic

Table 1. Response of chickens to H9N2 and H5N1 reassortant viruses.

Virus	Route	Morbidity (no. ill/total)	Mortality (no. dead/total)	Virus isolation		Seroconversion (AGP)
				Oropharyngeal swab	Cloacal swab	
PR8	i.n.	0/8	0/8	0/8[A]	1/8	3/8[B]
	i.v.	1/8	1/8 (3)[C]	0/8	8/8	7/7
Transfectant PR8	i.n.	0/8	0/8	0/8	0/8	0/8
	i.v.	0/8	0/8	1/8	6/8	7/8
H9N2/PR8	i.n.	0/8	0/8	8/8	0/8	8/8
	i.v.	0/8	0/8	4/8	7/8	8/8
G9	i.n.	0/8	0/8	7/8	7/8	8/8
	i.v.	0/8	0/8	8/8	8/8	8/8
H5N1/PR8	i.n.	0/8	0/8	0/8	0/8	0/8
	i.v.	0/8	0/8	0/8	0/8	4/8
HK491	i.n.	8/8	8/8 (1.9)	8/8	8/8	NA
	i.v.	8/8	8/8 (1.0)	8/8	8/8	NA

[A]Virus isolation positive/total chickens.
[B]AGP positive/total chickens.
[C]Mean time to death in days.

examination or for demonstration of influenza nucleoprotein by immunohistochemistry. Fifty percent chicken infectious doses (CID_{50}) were determined by infecting 10 chickens (4-week-old white leghorn or white rock) i.n. with 0.1 ml of serially diluted viruses. The birds were observed for 14 days, serum harvested, and tested by AGP for serologic evidence of infection. The CID_{50} was calculated by the method of Spearman and Karber (13).

Histopathology and immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. For detection of influenza A antigen, samples were stained immunohistochemically using a monoclonal antibody against influenza A virus NP as described previously (11).

RESULTS AND DISCUSSION

Pathogenicity of reassortant viruses. Wild type (wt) G9 virus caused no deaths, but the birds were clinically warmer than controls. In contrast, all chickens inoculated with HK491 died within 2 days p.i. One death was observed at day 3 p.i., in a chicken inoculated i.n. with PR8. The only clinical sign of illness, death, or gross lesions observed in chickens that were administered H5N1/PR8 or H9N2/PR8 reassortant viruses either i.n. or i.v. was in one of two chickens inoculated with H9N2/PR8 i.v. that showed mild tracheitis. Thus, neither reassortant virus was lethal to chickens (Table 1).

Virus isolation, histopathology, and serology. No virus was isolated from either oropharyngeal or cloacal swabs from chickens inoculated with wt PR8 or transfectant PR8 by the i.n. route. No viral antigen was demonstrated in tissues. Three of eight chickens inoculated with transfectant PR8 seroconverted but none that were inoculated with wt PR8. The results suggest that PR8 does not replicate efficiently in chickens. Viruses were isolated from cloacal swabs ($10^{1.9}$–$10^{5.1}$ EID_{50}/ml for transfectant PR8 and $10^{1.5}$–$10^{5.5}$ EID_{50}/ml for wt PR8) from chickens inoculated i.v. Most chickens seroconverted following i.v. inoculation.

When the H9N2 reassortant was given i.n., virus was isolated in low titers ($10^{1.5}$–$10^{3.5}$ EID_{50}/ml) from oropharyngeal swabs of eight of eight chickens, but not from cloacal swabs. No gross or histologic lesions were observed. On i.v. inoculation, mild tracheitis was seen in one of two chickens, with moderate amounts of antigen in the tracheal respiratory epithelium. Low titers of virus were recovered from oropharyngeal swabs of four of eight chickens ($10^{2.1}$–$10^{3.5}$ EID_{50}/ml) and from cloacal swabs from seven of eight chickens ($10^{0.97}$–$10^{4.5}$ EID_{50}/ml). All chickens inoculated either i.n. or i.v. seroconverted. Wt G9 virus was isolated in moderate titers from oropharyngeal ($10^{4.3}$–$10^{6.5}$ EID_{50}/ml with i.n. and $10^{3.3}$–$10^{6.1}$ EID_{50}/ml with i.v.) and cloacal swabs ($10^{0.97}$–$10^{2.3}$ EID_{50}/ml with i.n. and $10^{3.3}$–$10^{5.3}$ EID_{50}/ml with i.v.) and all chickens

seroconverted (Table 1). A mild nonspecific response of the monocyte-phagocytic system was seen in the spleen. Mild tracheitis and severe nephrosis with abundant viral antigen in necrotic tubules were also noted in some chickens with i.v. inoculation. Thus, the H9N2 reassortant has a different tissue tropism and replication pattern than the wt G9 virus and replication of reassortant virus was 10- to 100-fold lower than wt virus. The titers were similar to those from PR8 inoculated chickens.

No virus was isolated from oropharyngeal or cloacal swabs of chickens inoculated i.n. or i.v. with H5N1 reassortant virus. While four out of eight chickens that received i.v. inoculation seroconverted, none that received i.n. administered virus seroconverted. In contrast, all chickens died with either i.n. or i.v. inoculation of the HK491 virus. The wt H5 virus produced lesions typical of highly pathogenic avian influenza viruses in most visceral organs, including severe interstitial edema and pneumonia, necrosis of adrenal corticotrophic cells, cardiac myocyte degeneration and necrosis, and severe lymphocyte depletion and apoptosis in the spleen. Viral antigen was abundant in endothelial cells of blood vessels throughout all organs, cardiac myocytes, adrenal corticotrophic and chromaffin cells, microglial cells and neurons in the brain, and inflammatory cells. Virus was isolated in high titers from all oropharyngeal and cloacal swabs. Thus, unlike the H5N1 wt virus, the H5N1 reassortant replicated poorly in chickens and demonstrated a replication pattern similar to that of PR8.

Infectivity of reassortant viruses in chickens. The susceptibility of chickens to newly generated avian–human reassortant viruses was assessed by determining the CID_{50}. The CID_{50} of H5N1/PR8 and wt PR8 viruses were much higher than that of wt H5 virus requiring more than 1000 times higher titer of H5/PR8 virus to infect chickens. The CID_{50} of H9N2/PR8 was between those of transfectant PR8 and G9. The CID_{50} of either reassortant viruses was at least 10 times higher than the virus titer recovered from cloacal or oropharangeal swabs of chickens experimentally infected with $10^{6.0}$ of reassortant viruses. Therefore, it is unlikely that these reassortant viruses will pose a significant risk to poultry.

Candidate vaccine viruses, H5N1/PR8 and H9N2/PR8, were tested for their safety in poultry. Neither virus exhibited significant virulence, and both were poorly infectious in chickens. It is unlikely that these vaccine candidates will pose a biological threat to agriculture or the environment

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